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Probucol, a lipid-lowering drug, prevents cognitive and hippocampal synaptic impairments induced by amyloid β peptide in mice

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by synaptic loss and cognitive impairments. The presence of extracellular senile plaques (mainly composed of amyloid-β (Aβ) peptide) is an important molecular hallmark in AD and neuronal damage has been attributed, at least in part, to Aβ-mediated toxicity. Although the molecular mechanisms involved in the pathogenesis of AD are not yet completely understood, several lines of evidence indicate that oxidative stress and cholesterol dyshomeostasis play crucial roles in mediating the synaptic loss and cognitive deficits observed in AD patients. This study evaluated the effects of Probucol, a phenolic lipid-lowering agent with anti-inflammatory and antioxidant properties, on biochemical parameters related to oxidative stress and synaptic function (hippocampal glutathione and synaptophysin levels; glutathione peroxidase, glutathione reductase and acetylcholinesterase activities; lipid peroxidation), as well as on behavioral parameters related to the cognitive function (displaced and new object recognition tasks) in Aβ-exposed mice. Animals were treated with a single intracerebroventricu- ucol (10 mg/kg, i.p.) showed a significant hippocampal synaptophysin levels, as well as by an increase in hippocampal acetylcholinesterase activity. Importantly, Probucol treatment blunted the deleterious effects of $A\beta_{1-40}$ on learning-memory ability and hippocampal biochemistry. Although $A\beta_{1-40}$ treatment did not change hippocampal glutathione levels and glutathione peroxidase (GPx) and glutathione reductase (GR) activities, $A\beta_{1-40}$-exposed animals showed increased hippocampal lipid peroxidation and this event was completely blunted by Probucol treatment. These findings reinforce and extend the notion of the hazardous effects of $A\beta_{1-40}$ toward hippocampal synaptic homeostasis and cognitive functions. In addition, the present results indicate that Probucol is able to counteract the cognitive and biochemical impairments induced by i.c.v. $A\beta_{1-40}$ administration in mice. The study is the first to report the protective effects of Probucol (a "*non-statin cholesterol-lowering drug*") against $A\beta_{1-40}$-induced synaptic and behavioral impairments, rendering this compound a promising molecule for further pharmacological studies on the search for therapeutic strategies to treat or prevent AD.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive deficits associated with neuronal loss, which has been reported to occur as the result of interrelated events like oxidative stress, energetic dyshomeostasis, excitotoxicity and neuroinflammation (Butterfield and Lauderback, 2002; Kincses et al., 2010; Mattson, 2004a,b). These events have been connected, at least in part, to the extracellular deposition of amyloid β peptide (Aβ) and protein tau hyperphosphorylation in brain areas involved in cognitive functions (Armstrong, 2011; Braak and Braak, 1997;

Abbreviations: Aβ, amyloid-beta peptide; AD, Alzheimer's disease; PB, Probucol; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; i.c.v., intracerebroventricular; TBARS, thiobarbituric acid reactive substances; AChE, acetylcholinesterase; CNS, central nervous system.

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Hardy and Higgins, 1992; Mattson, 2004a,b), although the "*cause–consequence relationship*" between these events is not completely understood.

Aβ toxicity is likely to be mediated by multiple different Aβ forms, which occur as products of the activity of different proteases upon the amyloid precursor protein (APP) (Herring et al., 2011). Aβ can misarrange into soluble synaptotoxic oligomers (Haass and Selkoe, 2007; Shankar et al., 2008), as well as insoluble, pre-fibrillar and fibrillar aggregates, which finally precipitate as senile plaques (Walsh and Selkoe, 2004). Of particular importance, *in vitro* studies have shown that $A\beta_{1-40}$ can liberate hydroxyl radicals upon reaction with Fe(II), pointing to hydrogen peroxide generation as an important molecular mechanism mediating Aβ toxicity (Tabner et al., 2002). In agreement, *in vivo* studies have shown that amyloid plaques produce reactive oxygen species in living, Alzheimer's models and in human Alzheimer tissues (McLellan et al., 2003).

In addition to the extensively studied amyloid theory, which identifies Aβ as a critical molecule involved in the pathogenesis of AD (Hardy, 2009), accumulated evidence indicates that excessive cholesterol in the brain also plays important roles in the development and progression of this disorder (Longenberger and Shah, 2011). It is noteworthy that Aβ aggregation and brain cholesterol metabolism seem to be events that affect each other; increased cholesterol levels play an important role in mediating Aβ aggregation and cholesterol-lowering agents, such as HMG-CoA reductase inhibitors, may reduce Aβ accumulation by lowering brain cholesterol levels (Longenberger and Shah, 2011). Furthermore, clinical studies suggest that statins, which are the most potent agents for reduction of serum cholesterol (Gotto, 2002), via HMG-CoA reductase inhibition (Miida et al., 2007), may reduce risk and progression of AD (Haag et al., 2009). Possible additional mechanisms associated with these effects of statins include their "pleiotropic" effects, related to the modulation of inflammatory, vascular and immunological events (Kalayci et al., 2005; Pallebage-Gamarallage et al., 2010). Of particular importance, a recent study showed that atorvastatin prevented hippocampal cell death, neuroinflammation and oxidative stress induced by intracerebroventricular (i.c.v.) administration of aggregated $A\beta_{1-40}$ in mice (Piermartiri et al., 2010), suggesting that statins not only prevent Aβ aggregation (as reported by Longenberger and Shah, 2011), but also play protective effects by decreasing the toxicity induced by aggregated Aβ.

Probucol is a phenolic lipid-lowering prototype agent with anti-inflammatory and antioxidant properties that has a long history of clinical application for the treatment and prevention of cardiovascular diseases (Yamashita et al., 2008; Yamashita and Matsuzawa, 2009). Of particularly importance, a previous experimental study proposed that Probucol might play beneficial roles in inhibiting pathological processes that could be connected to the pathogenesis of AD (Champagne et al., 2003). That study (Champagne et al., 2003) showed that Probucol administration to aged rats induced the synthesis of hippocampal apolipoprotein E (apoE) and one of its main receptors (low density lipoprotein receptor-related protein; LRP), substantially attenuating age-related increases in glial activation and inducing synaptogenesis and dendritic remodeling. The results derived from such study (Champagne et al., 2003) are important due to the critical role of apoE in the pathogenesis of AD (Verghese et al., 2011), as well as due to the role of LRP in mediating brain-to-blood Aβ clearance (Zlokovic et al., 2010).

As mentioned previously, oxidative stress, neuroinflammation and brain cholesterol dyshomeostasis seem to mediate the development and progression of AD (Axelsen et al., 2011; Butterfield and Lauderback, 2002; Di Paolo and Kim, 2011; Mattson, 2004a,b). On the other hand, Probucol, a lipid-lowering agent with anti-inflammatory and antioxidant properties (Pallebage-Gamarallage et al., 2010), has been reported to play protective effects in experimental models of neurotoxicity/neuropathology (Farina et al., 2009; Park et al., 2007), as well as attenuated age-related increases in glial activation and induced synaptogenesis and dendritic remodeling in rats (Champagne et al., 2003). However, there are no studies on the potential relationship between Probucol and Aβ-induced neurotoxicity. Thus, we tested the hypothesis whether Probucol could inhibit the potential detrimental effects of $A\beta_{1-40}$ in the mouse hippocampal parameters and behavioral performance. We took advantage of a protocol previously standardized in our institute (Figueiredo et al., 2011; Medeiros et al., 2007), which shows that a single i.c.v. $A\beta_{1-40}$ injection causes inflammatory responses and synaptic changes associated with deficits on learning and memory. Biochemical parameters related to hippocampal oxidative stress and synaptic functions, as well as behavioral parameters related to the cognitive ability, were evaluated in an attempt to elucidate potential events mediating damage and neuroprotection.

Material and methods

Chemicals

Probucol, β-Nicotinamide adenine dinucleotide phosphate sodium salt reduced form, 5-5′-dithio-bis (2-nitrobenzoic) acid, glutathione reductase from baker's yeast, reduced glutathione and dimethyl sulfoxide were obtained from Sigma (St. Louis, MO, USA). Human $A\beta_{1-40}$ and $A\beta_{40-1}$ peptides were purchased from Innovagen (Lund, Sweden). All other chemicals were of the highest grade available commercially.

Animals

Adult Swiss male mice (90 days old), from our own breeding colony, were maintained at 22 °C, on a 12 h light:12 h dark cycle, with free access to food and water. All experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology (1989) and were approved by our ethics committee for animal use at the Universidade Federal de Santa Catarina (PP00326/CEUA 23080.013800/2009-90/UFSC).

Drug treatment protocol

Human $A\beta_{1-40}$ and $A\beta_{40-1}$ (reverse peptide) were prepared as stock solutions at a concentration of 1 mg/mL in sterile 0.1 M sodium phosphate-buffered saline (PBS) (pH 7.4), followed by aggregation by incubation at 37 °C for 4 days. $A\beta_{1-40}$ aggregation was confirmed by polyacrylamide gel electrophoresis (data not shown). The aggregated $A\beta_{1-40}$ (400 pmol/mouse), $A\beta_{40-1}$ (400 pmol/mouse) or PBS (vehicle) was administered i.c.v. as previously described (Medeiros et al., 2007; Prediger et al., 2007). These studies, as well as the validation protocol developed by Takeda et al. (2009), show that i.c.v. injection of $A\beta_{1-40}$ causes specific dysfunction of memory processing, which correlates well with the episodic memory deficit observed in the early stages of AD. Briefly, the animals were anesthetized with isoflurane (1 mL/mL; Abbot Laboratórios do Brasil Ltda., RJ, Brazil) using a vaporizer system (SurgiVet Inc., WI, USA) and then gently restrained by hand for i.c.v. injections. The sterilization of the injection site was carried out using gauze embedded in 70% ethanol. Under light anesthesia (*i.e.* just that necessary for loss of the postural reflex), the needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye and 1 mm posterior to a line drawn through the anterior base of the eyes (used as external reference). A volume of 3 μL of $A\beta_{1-40}$, $A\beta_{40-1}$ or PBS solution was injected into the lateral ventricle, at the following coordinates from bregma:anteroposterior (AP) = −0.1 mm, mediolateral (ML) = 1 mm, and dorsoventral (DV) = −2.4 mm.

Probucol was dissolved in saline (NaCl 0.9%) containing 10% of dimethyl sulfoxide (DMSO). To investigate the effect of long-term administration of Probucol on the cognitive and synaptic impairment, animals received 10 mg/kg of Probucol intraperitoneally (2 mL/kg;

i.p.) (Siveski-Iliskovic et al., 1995), once a day during two consecutive weeks (first administration 1 h after $A\beta_{1-40}$, $A\beta_{40-1}$ or PBS injections). Groups treated with vehicle (NaCl 0.9% with 10% DMSO, i.p.) were used as control.

Behavioral analysis

Twenty four hours after the last Probucol administration, displaced and new object recognition tasks were conducted for evaluation of learning-memory ability. The apparatus, objects and procedures were based on a previously standardized protocol (Thinus-Blanc et al., 1996). The apparatus was a blue plastic circular open field ($d = 44$ cm; $h = 22$ cm) with sectors drawn on the floor. The objects were: two identical triangular rubber stoppers brown ($l = 9$ cm; $w = 6.5$ cm; $h = 12$ cm); and the object that was used to test object novelty was a conical tube striped yellow and white ($l = 9$ cm; $w = 6.5$ cm; $h = 12$ cm). Each mouse was submitted to four consecutive sessions (6 min in duration; 3 min intersession interval).

The first session was used to assess locomotor activity (no objects present) and total number of crossings and rearings were recorded. During sessions 2, 3 and 4, two objects were presented in the open field with visual clues, composed by a black and white vertical striped panel attached to the wall. In session 2, the mice were placed in the center of the apparatus with two identical objects. Later, in the session 3, one object was moved to a new location and the time spent exploring the displaced object (new location) and the non-displaced object (old location) was recorded. In order to analyze the cognitive performance, a location index was calculated using $(T_{displaced})/(T_{displaced} + T_{non\text{-}displaced})$, where $T_{displaced}$ is the time spent exploring the displaced object and $T_{non\text{-}displaced}$ is the time spent exploring the non-displaced object, as previously described (Murai et al., 2007). In session 4, a new object was added in the apparatus (replacing one of the two familiar objects) and the time spent exploring the new object and the familiar object (old object) was calculated using $(T_{new})/(T_{new} + T_{familiar})$, where T_{new} is the time spent exploring the new object and $T_{familiar}$ is the time spent exploring the old object (Hyde and Crnic, 2002). After each session, the experimental apparatus was cleaned only with dry paper thus minimizing the interference of potential unfamiliar smells. Five/four animals of each group were used for biochemical analysis, while 3–4 animals were intracardiacly perfused with saline and 4% of the fixative paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) for immunohistochemistry analysis.

Tissue preparation for biochemical analyses

Twenty four hours after of the behavioral analyses, animals (4–5 per group) were submitted to anesthesia, as described above, and the blood was collected by cardiac puncture in heparinized tubes. Then, the animals were killed by decapitation and hippocampus was removed and homogenized (1:10 w/v) in HEPES buffer (20 mM, pH 7.0). The tissue homogenates were centrifuged at $3000 \times g$, at 4 °C for 5 min and an aliquot of the low-speed supernatant was used for the determination of acetylcholinesterase (AChE) activity. Thereafter, the supernatants of the first centrifugation were further centrifuged at $16{,}000 \times g$, at 4 °C for 20 min and the supernatants obtained were used for the determination of enzymatic activities and for the quantification of the levels of glutathione (GSH) and thiobarbituric acid reactive substances (TBARS). Whole blood was centrifuged at $3000 \times g$, at room temperature for 10 min and the obtained plasma was used to measure total cholesterol levels.

Biochemical analyses

Acetylcholinesterase (AChE) activity

Hippocampal AChE activity was measured as previously described (Ellman et al., 1961), using acetylthiocholine iodide as a substrate. The rate of hydrolysis of acetylthiocholine iodide was measured at 412 nm through the release of the thiol compound (thiocholine), which produces the color-forming compound TNB after reaction with DTNB.

Antioxidant enzymes

Hippocampal glutathione reductase (GR) activity was measured using an NADPH reduction assay following the protocol developed by Carlberg and Mannervik (1985) using glutathione disulfide (GSSG) as substrate. GR activity was monitored by decreases in NADPH absorbance at 340 nm at 37 °C in a TECAN Genios Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). Results were based on a molar extinction coefficient for NADPH of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$. Hippocampal glutathione peroxidase (GPx) activity was measured using an NADPH reduction assay following the technique of Wendel (1981). Tissue supernatant (around 200 μg protein) was added to a reaction mixture containing reduced glutathione, glutathione reductase, and NADPH in phosphate buffer (pH 7.4). The reaction was initiated by adding *tert*-butyl hydroperoxide, the absorbance decrease at 340 nm was recorded at 37 °C in a TECAN Genios Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). The activity in the absence of the samples was subtracted. Results were based on a molar extinction coefficient for NADPH of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$.

Cholesterol levels

Total cholesterol levels were determined in plasma by an enzymatic method based on the oxidase/peroxidase system using commercial kit reagents (Labtest Diagnostica®, Lagoa Santa-MG, Brazil).

Glutathione levels

Hippocampal glutathione (GSH) levels were measured as non-protein thiols based on the protocol developed by Ellman (1959). Hippocampal homogenates were precipitated in cooled trichloroacetic acid 10% and centrifuged at $5000 \times g$ for 10 min, and the supernatant was incubated with DTNB in a 1 M phosphate buffer, pH 7.0. Absorbance was measured at 412 nm. A standard curve of reduced glutathione was used to calculate GSH levels.

Determination of thiobarbituric acid reactive substances levels

Thiobarbituric acid reactive substances (TBARS) were determined in the hippocampal homogenates using the method described by Ohkawa et al. (1979), in which malondialdehyde (MDA), an end-product of lipid peroxidation, reacts with thiobarbituric acid to form a colored complex. The samples were incubated at 100 °C for 60 min in acid medium containing 0.45% sodium dodecyl sulfate and 0.67% thiobarbituric acid. After centrifugation, the reaction product was determined at 532 nm using MDA as standard.

Protein determination

The protein content was quantified by the method of Bradford (1976), using bovine serum albumin as a standard.

Immunohistochemistry for synaptophysin

Immunohistochemical procedures were carried out on paraffin-embedded brain tissue sections using monoclonal mouse anti-synaptophysin (1:400; Novocastra, Newcastle, UK), as previously described (Figueiredo et al., 2011). Briefly, following quenching of endogenous peroxidase with 1.5% hydrogen peroxide in methanol (v/v) for 20 min, high temperature antigen retrieval was performed by immersion of the slides in a water bath at 95–98 °C in 10 mM trisodium citrate buffer pH 6.0, for 45 min. Then, the slides were processed using the appropriate biotinylated secondary antibody, and the streptavidin-HRP complex (Vector Laboratories, Burlingame, CA), and according to the manufacturer's instructions. Subsequently, the sections were developed with DAB (3,3′-diaminobenzidine) (Dako Cytomation, Carpinteria, CA) in

chromogen solution and counterstained with Harris hematoxylin. All tissue samples were equally and simultaneously processed during all immunohistochemistry assays, and, importantly, remained under chromogen exposition for the same period of time.

Image analysis

The immunostaining was assessed at the dorsal hippocampus, in 3-μm sections obtained between 1.6 and 2.4 mm posterior to the Bregma. Images of stained hippocampal CA1, CA2, CA3 and dentate gyrus (DG) were acquired using a Sight DS-5M-L1 digital camera (Nikon, Melville, NY, USA) connected to an Eclipse 50i light microscope (Nikon) at 400× magnification. Four images of each hippocampal section were captured according to previously described (Medeiros et al., 2007). The windows (ROI) for synaptophysin quantification were positioned in the CA1 and CA2 stratum radiatum, CA3 stratum lucidum, and DG polymorph layer subregions. A threshold optical density that best discriminated staining from the background was obtained using the NIH ImageJ 1.36b imaging software (NIH, Bethesda, MD, USA), and total pixels intensity was determined and data were expressed as optical density (O.D.). The data represent the average value obtained by the analysis of images of the hippocampal CA1, CA2, CA3 and dentate gyrus.

Statistical analysis

Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Differences among the five groups (including the negative control; reverse peptide) were analyzed by one-way ANOVA followed by the Tukey *post hoc* test. Alternatively, $A\beta_{1-40}$ *vs.* Probucol interactions (excluding the negative control; reverse peptide) were analyzed by two-way ANOVA followed by the Bonferroni *post hoc* test. Results are expressed as mean ± SEM. The differences were considered significant when $p<0.05$.

Results

Probucol treatment decreases plasma cholesterol levels and did not affect general health in mice

No significant differences among groups were observed in general health parameters, such as body weight, food and liquid consumption (data not shown). Total cholesterol levels significantly decreased in the plasma of animals treated with Probucol (confirming its hypocholesterolemic effects), independently from $A\beta_{1-40}$ administration [$F_{4,32}=17.68$, $p<0.001$] (Fig. 1). The i.c.v. $A\beta_{1-40}$ treatment did not change total cholesterol levels in plasma (Fig. 1).

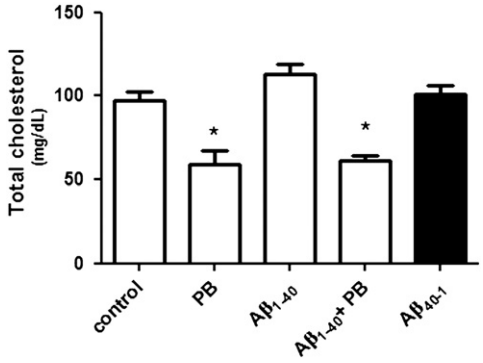

Fig. 1. Probucol (PB) reduces plasma cholesterol levels in mice. The animals were submitted to i.c.v. administration of $A\beta_{1-40}$, $A\beta_{40-1}$ or PBS and treated intraperitoneally with Probucol (10 mg/kg) or vehicle, once a day, during 15 consecutive days. Plasma cholesterol levels are expressed as mg/dL and presented as mean ± S.E.M. (n = 7–9 mice/group). All the five groups (including the negative control; reverse peptide) were compared for significant differences by analysis of variance (ANOVA). *$p<0.05$ when compared with the control group by one-way ANOVA followed by Tukey's multiple comparison test.

Probucol treatment improves the cognitive impairment induced by $A\beta_{1-40}$

In order to evaluate the effects of i.c.v. $A\beta_{1-40}$ injection on mouse cognitive performance, a two-trial object recognition task was used (see Material and methods section). $A\beta_{1-40}$ administration caused a significant decline in the spatial memory [$F_{4,32}=4.25$; $p<0.01$], as indicated by a decrease in the time spent in the displaced object during the 3rd test session when compared to controls (Fig. 2C). Moreover, $A\beta_{1-40}$ administration caused in a significant decline in the capacity of recognizing a previous presented experience [$F_{4,32}=3.723$; $p<0.05$], as indicated by a decrease in the time exploring the new object during the 4th test session when compared to controls (Fig. 2D). Probucol treatment prevented the $A\beta_{1-40}$-induced cognitive deficits in both tests sessions (Figs. 2C and D) and no cognitive deficits were observed in mice treated with the inverse peptide ($A\beta_{40-1}$; negative control). No significant differences in the number of crossed squares and rearing behavior were observed among the experimental groups (Figs. 1A and B), indicating that the changes observed in the object recognition tasks were not related to motor impairment (Figs. 2A and B).

Probucol treatment improves the synaptic deficits induced by $A\beta_{1-40}$

To further explore potential events involved in the $A\beta_{1-40}$-induced cognitive deficits, we assessed the levels of the presynaptic protein synaptophysin in the hippocampus 15 days after the beginning of treatments (24 h after the behavioral sessions). The $A\beta_{1-40}$ treatment resulted in a significant loss of synaptic marker synaptophysin in the mouse hippocampus and Probucol treatment significantly attenuated this effect [$F_{4,11}=4.976$, $p<0.05$] (Fig. 3). These data suggest that Probucol might protect against the $A\beta_{1-40}$-induced cognitive failure through a reduction of the synaptic changes induced by the peptide.

Probucol treatment prevents the increase in acetylcholinesterase (AChE) activity induced by $A\beta_{1-40}$

$A\beta_{1-40}$ injection significantly increased hippocampal AChE activity 15 days after the peptide administration [$F_{4,18}=4551$; $P<0.05$] and Probucol, which did not affect AChE activity *per se*, blunted the stimulatory effect played by $A\beta_{1-40}$. The inverse peptide ($A\beta_{40-1}$; negative control) did not change hippocampal AChE activity (Fig. 4).

Probucol treatment modulates the hippocampal glutathione reductase activity

The antioxidant glutathione (GSH) system is an important tool mediating protection against several (pro)-oxidant molecules in the CNS (Dringen and Hirrlinger, 2003) and the dyshomeostasis of this system is involved in several neuropathological conditions (Lee et al., 2010; Lovell et al., 1998). Probucol treatment caused a significant increase in hippocampal glutathione reductase (GR) activity as indicated by a significant main effect in a two-way ANOVA ($A\beta_{1-40}$ and Probucol as independent variables) [$F_{1,15}=6.059$, $P<0.05$] (Fig. 5A). Moreover, one-way ANOVA showed a significant increase in hippocampal GR activity in animals treatment with Probucol (i.p.) and in animals treatment with Probucol (i.p) plus $A\beta_{1-40}$ (i.c.v.) [$F_{4,18}=4.551$, $P<0.05$] (Fig. 5A). Hippocampal GSH content and glutathione peroxidase (GPx) activity were not significantly different among groups (data not shown).

Hippocampal lipid peroxidation increased in $A\beta_{1-40}$-treated animals [$F_{4,18}=2.525$, $P=0.07$] and Probucol treatment blunted this event (Fig. 5B). It is noteworthy that animals treated with the inverse peptide ($A\beta_{40-1}$) showed a similar hippocampal lipid peroxidation to

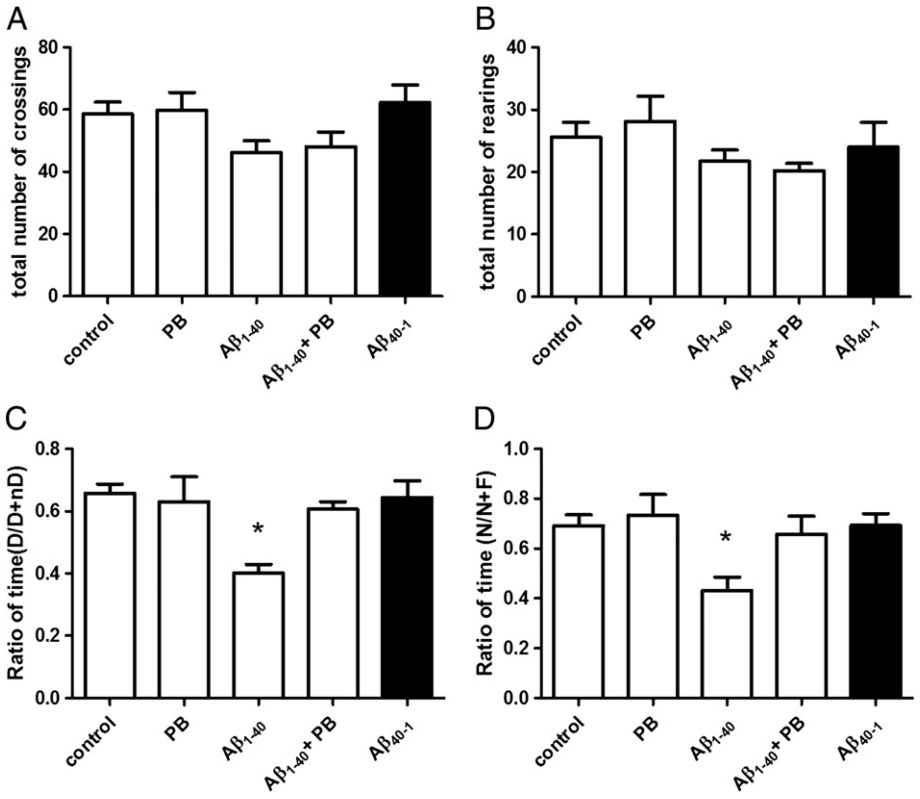

Fig. 2. Probucol (PB) attenuates cognitive impairment induced by $A\beta_{1-40}$ in mice. The animals were submitted to i.c.v. administration of $A\beta_{1-40}$, $A\beta_{40-1}$ or PBS and treated intraperitoneally with Probucol (10 mg/kg) or vehicle, once a day, during 15 consecutive days. Locomotor (A) and exploratory (B) activities, as well as the recognition of displaced (C) and new (D) objects, were evaluated at 24 h after the last Probucol administration. The results are expressed as the total number of crossings (A), total number of rearings (B), the ratio between times spent in the displaced (C) or new (D) objects and the total time spent in both objects (displaced + nondisplaced or new + familiar). Data are presented as mean ± S.E.M. (n = 7–9 mice/group). All the five groups (including the negative control; reverse peptide) were compared for significant differences by analysis of variance (ANOVA). *$p<0.05$ when compared with the control group by one-way ANOVA followed by Tukey's multiple comparison test.

that of control animals (Fig. 5B). A significant positive correlation was found for hippocampal TBARS levels and AChE activity [$r = 0.66$, $P<0.01$].

Discussion

The present results show that a two-week treatment of adult mice with Probucol, a phenolic lipid-lowering agent with anti-inflammatory and antioxidant properties, protected against behavioral and hippocampal biochemical changes induced by a single i.c.v. administration of $A\beta_{1-40}$, significantly attenuating $A\beta_{1-40}$-induced impairments in the object recognition memory and hippocampal lipid peroxidation, synaptic dyshomeostasis and changes in acetylcholinesterase activity. To the best of our knowledge, this is the first *in vivo* study reporting the beneficial effects of this "*non-statin*" cholesterol-lowering drug in an experimental model of AD based on Aβ-mediated neurotoxicity.

Loss of memory and impairment of cognitive functions represent classical signs observed in AD patients (Mattson, 2004a,b), which have been directly correlated with Aβ deposition (Cummings et al., 1996). Our experimental protocol (based on the i.c.v. infusion of $A\beta_{1-40}$ in mice) caused a significant decline in the object recognition memory, which is highly dependent upon the hippocampal system (Broadbent et al., 2010). Importantly, the i.c.v. infusion of $A\beta_{40-1}$ (reverse peptide) did not affected the ability of animals in recognizing both the *new* and *replaced* objects, indicating that the observed behavioral impairments in $A\beta_{1-40}$-exposed animals were dependent on the peptide sequence/structure, supporting the relevance of the chosen experimental model (Figueiredo et al., 2011; Medeiros et al., 2007; Piermartiri et al., 2010). It is noteworthy that this protocol also caused significant cognitive impairments in mice when evaluated in others cognitive tasks (*i.e.*, Moris water maze test; Medeiros et al., 2007; Prediger et al., 2007), reinforcing the importance of a potential relationship between cognitive behavior and synaptic function in our experimental model.

Synapse loss at specific encephalic structures of AD patients significantly correlates with the severity of their cognitive symptoms (Scheff et al., 2006). In our experimental model, i.c.v. $A\beta_{1-40}$ infusion significantly decreased the hippocampal levels of synaptophysin, the major synaptic vesicle protein (consequently, a specific pre-synaptic marker), although no effects were observed in animals exposed to the reverse peptide ($A\beta_{40-1}$). The decreased synaptophysin levels and the impaired object recognition memory in $A\beta_{1-40}$-exposed mice greatly suggest a link between both events; the synaptotoxic effects of $A\beta_{1-40}$ might be crucial in causing the observed memory deficits. This idea is reinforced by the fact that Probucol, which prevented $A\beta_{1-40}$-induced synaptic changes, also blunted the deleterious effects of $A\beta_{1-40}$ on the recognition object memory.

The actual mechanisms related to the beneficial effects of Probucol against $A\beta_{1-40}$-induced synaptic and cognitive impairments cannot be completely explained only based on the presented results. In fact, experimental and clinical studies have shown that Probucol decreases the progression of atherosclerosis (Regnstrom et al., 1996), protects vasculature (Poirier, 2003), acts as an antioxidant molecule (Singla et al., 2007), enhances neurogenesis (Champagne et al., 2003), inhibits thrombosis (Tanous et al., 2006), modulates the activity of antioxidant enzymes (Farina et al., 2009) and possesses anti-inflammatory properties (Pfuetze and Dujovne, 2000). Taking into account the numerous factors involved in the toxicity elicited by Aβ (*i.e.*, inflammation, oxidative stress, glutamate and calcium dyshomeostasis, modulation of cholesterol homeostasis — (Longenberger and Shah, 2011; Tabner et al., 2006), all the aforementioned Probucol properties

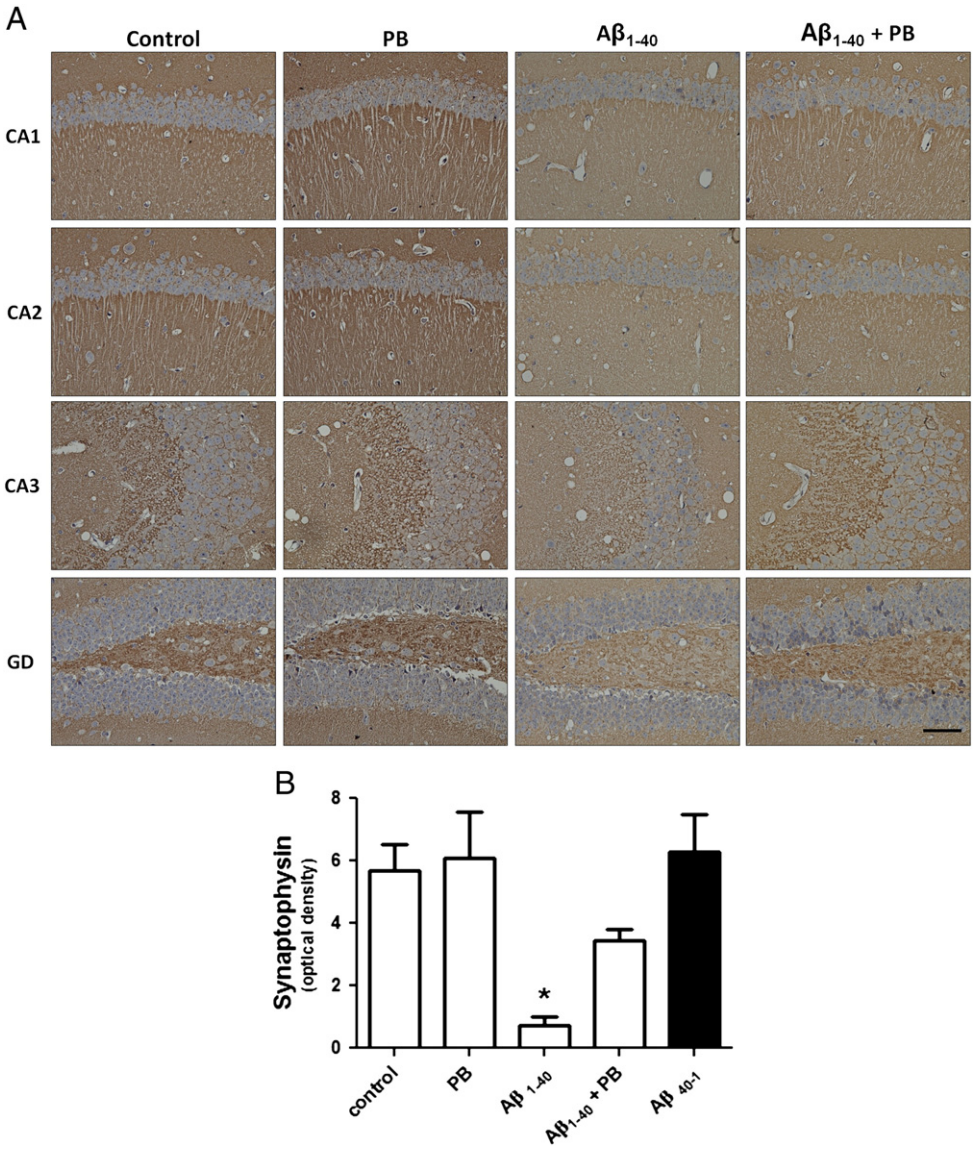

Fig. 3. Probucol (PB) prevents $A\beta_{1-40}$-induced synaptic disruption in mice. The animals were submitted to i.c.v. administration of $A\beta_{1-40}$, $A\beta_{40-1}$ or PBS and treated intraperitoneally with Probucol (10 mg/kg) or vehicle, once a day, during 15 consecutive days. (A) Representative images of synaptophysin immunoreactivity in CA1, CA2, CA3 and dentate gyrus (DG) subregions of mice hippocampus (scale bar = 50 μm). (B) Relative quantification of synaptophysin optical density in hippocampal subregions CA1, CA2, CA3, and DG. Values represent the mean ± S.E.M. (n = 3–5 mice/group). All the five groups (including the negative control; reverse peptide) were compared for significant differences by analysis of variance (ANOVA). *$p < 0.05$ when compared with the control group by one-way ANOVA followed by Tukey's multiple comparison test.

could contribute to its beneficial effects observed in our experimental model. Of particular importance, neuroinflammatory-like changes have been found in the hippocampus of rodent with memory impairment caused by Aβ administration (Medeiros et al., 2007). Because Probucol is an anti-inflammatory drug, the control of neuroinflammation might be a possible mechanism by which Probucol affords neuroprotection in our experimental protocol. In addition, it is important to note that the i.c.v. $A\beta_{1-40}$ infusion increased the levels of hippocampal lipid peroxidation and Probucol significantly blunted this phenomenon. These results suggest that oxidative stress played an important role in mediating the deleterious effects of $A\beta_{1-40}$ toward hippocampal synapses and that the antioxidant properties of Probucol were important in counteracting $A\beta_{1-40}$ effects. This idea is supported by studies reporting the pro-oxidative properties of $A\beta_{1-40}$ (Medeiros et al., 2007; Piermartiri et al., 2010; Tabner et al., 2006), as well as the antioxidant properties of Probucol (Farina et al., 2009; Park et al., 2007). Although Probucol treatment was able to increase hippocampal glutathione reductase (GR) activity, which could contribute to the observed beneficial effects against $A\beta_{1-40}$-mediated synaptic impairment, its direct scavenging abilities (Bridges et al., 1991) might also explain its positive effects in decreasing $A\beta_{1-40}$-induced hippocampal lipid peroxidation and, consequently, synaptotoxicity.

Although it is likely that Probucol's antioxidant properties contribute to its beneficial effects against $A\beta_{1-40}$-induced synaptic and cognitive impairments, such benefit could also be related to its modulatory effects toward cholesterol metabolism. Of particular importance, recent findings lend support to the notion that progressive deterioration of cholesterol homeostasis in AD is a central player in the disease pathophysiology (for a review, see Leduc et al., 2010). Cholesterol has been linked to the amyloidogenesis process (Guardia-Laguarta et al., 2009) and was shown to accumulate in senile plaques of AD patients and in transgenic mice (Mori et al., 2001). On the other hand, hypocholesterolemic agents have presented beneficial effects in experimental models of AD, as well as in clinical/epidemiological studies (Haag et al., 2009; Piermartiri et al., 2010). Of particular importance, statins, which represent widely used drugs that reduce cholesterol synthesis by

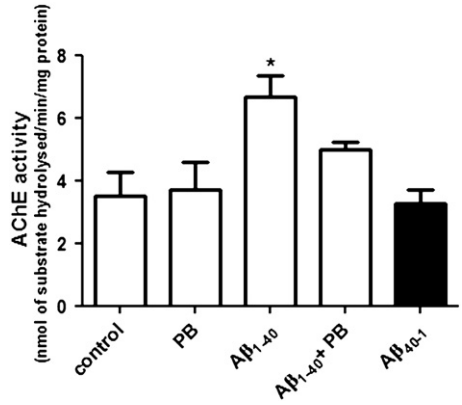

Fig. 4. Probucol (PB) prevents the increase of AChE activity induced by $A\beta_{1-40}$ in the hippocampus of mice. The animals were submitted to i.c.v. administration of $A\beta_{1-40}$, $A\beta_{40-1}$ or PBS and treated intraperitoneally with Probucol (10 mg/kg) or vehicle, once a day, during 15 consecutive days. Enzyme activity is expressed as nmol of substrate hydrolyzed/min/mg protein and presented as mean ± S.E.M. (n = 4–5 mice/group). All the five groups (including the negative control; reverse peptide) were compared for significant differences by analysis of variance (ANOVA). *$p < 0.05$ when compared with the control group by one-way ANOVA followed by Tukey's multiple comparison test.

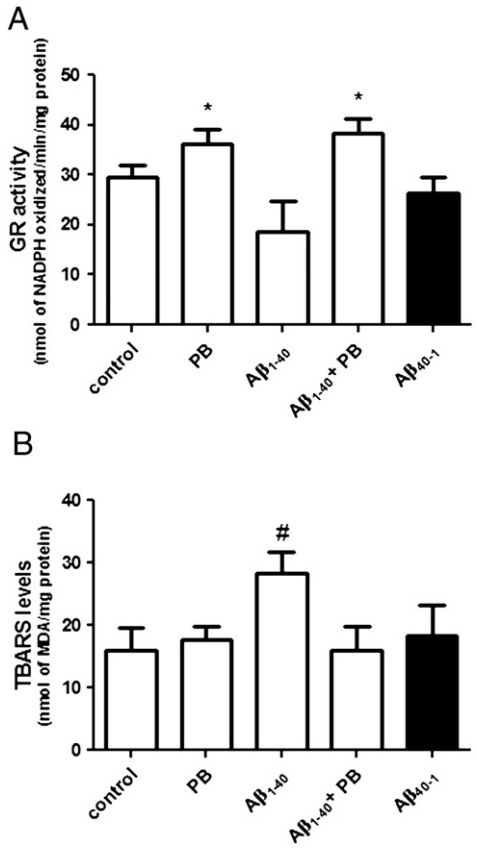

Fig. 5. Probucol (PB) prevents the decrease in glutathione reductase (GR) activity induced by $A\beta_{1-40}$ in the hippocampus of mice. The animals were submitted to i.c.v. administration of $A\beta_{1-40}$, $A\beta_{40-1}$ or PBS and treated intraperitoneally with Probucol (10 mg/kg) or vehicle, once a day, during 15 consecutive days. Hippocampal glutathione reductase (GR) activity (A) is expressed as nmol of NADPH oxidized/min/mg protein and thiobarbituric acid reactive substances (TBARS) levels (B) are expressed as nmol of MDA/mg protein. Data are presented as mean ± S.E.M. (n = 4–5 mice/group). All the five groups (including the negative control; reverse peptide) were compared for significant differences by analysis of variance (ANOVA). *$p < 0.05$ when compared with the control group by one-way ANOVA followed by Tukey's multiple comparison test. #$p = 0.07$ when compared with the control group by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

inhibiting 3-hydroxyl-3-methylglutaryl coenzyme A (HMG CoA) reductase (Jick et al., 2000; Rockwood et al., 2002), have been reported to reduce risk and progression of AD (Haag et al., 2009). A recent study showed that atorvastatin prevented hippocampal damage induced by aggregated $A\beta_{1-40}$ infusion (exposure schedule identical to ours) (Piermartiri et al., 2010), suggesting that statins not only prevent Aβ aggregation (as reported by Longenberger and Shah, 2011), but also play protective effects by decreasing the toxicity induced by aggregated Aβ. Interestingly, Probucol also protected against the toxic effects elicited by i.c.v. infused $A\beta_{1-40}$, suggesting that both hypocholesterolemic agents could act similarly in protecting against $A\beta_{1-40}$-toxicity. However, an additional advantage of Probucol when compared to statins could be its high antioxidant activity, which seems to protect against $A\beta_{1-40}$-induced oxidative damage. Moreover, experimental evidences indicate that Probucol increased the hippocampal levels of apoE and its main receptor (LRP) (Champagne et al., 2003), which is crucial in mediating brain-to-blood Aβ clearance (Zlokovic et al., 2010). Thus, one could suppose that, in addition to its antioxidant effects, Probucol might protect against $A\beta_{1-40}$-induced synaptic and cognitive impairments by modulating cholesterol metabolism, as well as apoE and LPR expression. However, additional studies are necessary to prove this hypothesis. The levels of plasma cholesterol were decreased in Probucol treated animals. Preliminary studies from our laboratory (not published) have shown that brain cholesterol levels are not significantly changed after Probucol treatment in mice. However, we do not know about the distribution of brain cholesterol into specific hippocampal membrane regions (particularly, lipid rafts) after Probucol treatment. This constitutes a very interesting field that deserves additional research.

Because dose–response *in vivo* studies concerning the hypocholesterolemic effects of Probucol in mice are lacking in the literature, we have no comparative basis to affirm whether the Probucol's dosage (10 mg/kg; during 2 weeks) used in our study represented a supra-maximal effective dose. Nevertheless, it was noteworthy that the chosen dosage (based on Siveski-Iliskovic et al., 1995) decreased around 45% of plasma total cholesterol levels, indicating a highly effective pharmacological effect. Although it is difficult to extrapolate our protocol to human conditions, similar doses have been used in clinical trials (Jeon et al., 2011; Kasai et al., 2011; Reaven et al., 1992), where the daily dose of 1 g and 500 mg for patients would be equivalent to approximately 14 mg/kg/day and 7 mg/kg/day, respectively, considering a mean body weight of 70 kg for an adult human. Interestingly, experimental studies with rabbits and rodents showed that a similar dose of Probucol (approximately 10 mg/kg) leaded to plasmatic Probucol concentrations ranging from 43 to 58 μg/mL, which are comparable to those found in the plasma of humans receiving a standard oral dose of 1 g of Probucol per day (Aburatani et al., 1988; Kita et al., 1988; Shankar et al., 1989).

The close relationship between the cholinergic system and the pathogenesis and symptoms of AD is a well reported phenomenon (Van Beek and Claassen, 2011). Of particular importance, a severe loss of cholinergic innervations has extensively been documented during the advanced stages of late-onset AD (Schliebs and Arendt, 2011). Acetylcholinesterase (AChE) inhibitors provide beneficial effects in AD patients (ameliorating cognitive skills), at least in the beginning of the progressive disease (Holzgrabe et al., 2007). Paradoxically, despite the overall loss of AChE in the brain of AD patients (Atack et al., 1983), AChE activity is increased in plaques and tangles very early in the process of amyloid deposition (Moran et al., 1993; Ulrich et al., 1990). Moreover, a recent study showed that AD patients presented a higher plasma AChE activity when compared to subjects of the same age (Garcia-Ayllon et al., 2010). Such event was correlated with an increase in the G(1) + G(2) forms, the subset of AChE species which are increased in brain of AD patients (Garcia-Ayllon et al., 2010). The mechanism by which AChE expression is upregulated around amyloid plaques is unclear, but appears to be due to a disturbance in calcium homeostasis (Sberna et al., 1997). Our results showed that i.c.v. $A\beta_{1-40}$ infusion increased hippocampal

AChE activity. In addition, Probucol treatment, which did not change AChE activity *per se*, blunted the stimulatory effect of $A\beta_{1-40}$ toward hippocampal AChE. Based on the potential involvement of calcium dyshomeostasis in the increased AChE activity near to amyloid plaques (Sberna et al., 1997) and the close relationship between calcium dyshomeostasis and oxidative stress (Lafon-Cazal et al., 1993), one could suppose a potential relationship between oxidative stress and increased AChE activity in the hippocampus of $A\beta_{1-40}$-exposed mice. Accordingly, experimental evidences have reported increased AChE activity in the CNS during oxidative stress events (Khadrawy et al., 2011). Probucol, whose beneficial effects against $A\beta_{1-40}$-induced synaptic and memory impairments seems to be related to its antioxidant properties, also decreased hippocampal AChE activity in $A\beta_{1-40}$-exposed mice. In this regard, it was noteworthy the observed significant positive correlation between hippocampal lipid peroxidation and AChE activity, reinforcing the potential relationship between oxidative stress and increased AChE activity (both events abolished by Probucol treatment).

In conclusion, the present findings reinforce and extend the notion of the hazardous effects of $A\beta_{1-40}$ toward hippocampal synaptic homeostasis and cognitive performance. In addition, they indicate that Probucol is able to counteract the behavioral and biochemical impairments induced by i.c.v. $A\beta_{1-40}$ administration in mice. The antioxidant properties of Probucol are likely related to its beneficial effects, although the involvement of cholesterol metabolism modulation in this benefit cannot be ruled out. The study is the first to report the protective effects of Probucol (a "*non-statin cholesterol-lowering drug*") against $A\beta_{1-40}$-induced synaptic and behavioral impairments, rendering this compound a promising molecule for further pharmacological studies on the search for therapeutic strategies to treat or prevent AD.

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